

Suppression of intratumoral CCL22 by type I interferon inhibits migration of regulatory T cells and blocks cancer progression

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Abstract

The chemokine CCL22 is abundantly expressed in many types of cancer and is instrumental for intratumoral recruitment of regulatory T cells (Treg), an important subset of immunosuppressive and tumor-promoting lymphocytes. In this study, we offer evidence for a generalized strategy to blunt Treg activity that can limit immune escape and promote tumor rejection. Activation of innate immunity with Toll-like receptor (TLR) or RIG-I-like receptor (RLR) ligands prevented accumulation of Treg in tumors by blocking their immigration. Mechanistic investigations indicated Treg blockade was a consequence of reduced intratumoral CCL22 levels caused by type I interferon. Notably, stable expression of CCL22 abrogated the antitumor effects of treatment with RLR or TLR ligands. Taken together, our findings argue that type I interferon blocks the Treg-attracting chemokine CCL22 and thus helps limit the recruitment of Treg to tumors, a finding with implications for cancer immunotherapy.

Introduction

The chemokine CCL22 is abundantly expressed in the tissue of many types of human cancer (1,2). Its cognate receptor is CCR4, a transmembrane protein expressed predominantly and constitutively by regulatory T cells (Treg), a subpopulation of immunosuppressive T lymphocytes (3). Several studies show that CCL22 leads to the recruitment of Treg to the cancer tissue (1,2). Although the role of Treg in cancer remains to be definitely established, many reports on human and murine cancers demonstrate a tumor-promoting effect: Treg progressively accumulate in the blood and lymphoid organs (4) and abundantly infiltrate the tumor tissue itself (5). These tumor-infiltrating Treg, that express CD25 and the transcription factor FoxP3 strongly suppress effector T cell function *ex vivo* (1). Indeed, suppression of anti-cancer immunity is mediated predominantly by intratumoral Treg that suppress CD8 T cell responses locally at the tumor site (6). High numbers of tumor-infiltrating FoxP3+ Treg correlate with poor prognosis and have been identified as a significant predictor of patient death in several types of human cancer (1,2).

The aim of cancer immunotherapy is to promote anti-tumor immunity and to overcome tumor-induced immunosuppression. Activation of the innate immune system with ligands for pattern-recognition receptors (PRR) or with cytokines such as IFN- α can suppress tumor growth (7,8). In mice, CpG oligonucleotides (CpG), that stimulate the TLR9 receptor, improve the efficacy of anti-cancer vaccines and can also be used as single agent to reduce tumor size (9,10). In humans, imiquimod, a synthetic agonist for TLR7, is used to treat basal cell carcinoma and vulvar intraepithelial neoplasia, and the cytokines IL-2 and IFN- α are used for selected patients with melanoma or renal cell cancer (11,12). Further, many TLR activators are currently under investigation in clinical trials (13).

The impact of PRR-activating ligands on Treg in the tumor-bearing host is still unclear. It has been shown that PRR activation can prevent Treg-mediated suppression of T effector cell proliferation in the lymph node (14,15), but an effect of PRR activation on Treg in the tumor tissue has so far not been described. In particular, it is unknown whether trafficking and tumor infiltration of Treg can be affected by agonists for TLRs and RIG-I-like helicases.

Materials and Methods

Mice and cell lines

Female BALB/c and C57BL/6 mice were from Harlan-Winkelmann (Borchen, Germany). IFN- γ receptor (IFNAR)-deficient mice on C57BL/6 background were kindly provided by Dr. Z. Waibler (Paul-Ehrlich Institute, Langen, Germany). Mice were 5 to 10 weeks of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The human cell lines Jurkat, A-375, A-431, A-549, CAMA-1, MCF-7, MDA-MB-231, MDA-MB-435s, PANC-1, SK-BR-3, SW480 and the murine cell lines 4T1, CT26, EL-4 (EG-7) and B16-F1 were obtained from American Type Culture Collection, where short tandem repeat (STR) analysis is used for authentication, and were used within 6 months after resuscitation (ATCC, Manassas, VA, USA). IMIM-PC1 was kindly provided by Prof. Michl (Marburg, Germany), Panc02 by Prof. Bruns, MethA by Prof. Zimmermann, and mGC8 by Dr. Nöckel (all Munich, Germany). Cell lines were authenticated using STR (LGC Standards) and were cultured in complete DMEM or RPMI medium (PAA Laboratories) and routinely tested for mycoplasma contamination by MycoAlert™ Mycoplasma Detection Kit (LONZA). The CT26-CCL22^{dox} cell line was generated by lentiviral transduction with a construct containing a doxycycline (Dox)-inducible CCL22 expression cassette as described (16). For tumor induction 0.25×10^6 (CT26 or CT26-CCL22^{dox}), 1×10^6 (B16 and Panc02) and 7.5×10^6 (EG-7) tumor cells were injected subcutaneously into the flank. Mice with subcutaneous CT26-CCL22^{dox} tumors were fed with a normal or 25 mg/kg doxycycline-containing diet (ssniff Spezialdiäten GmbH, Soest, Germany). Tumor size was expressed as the product of the perpendicular diameters of individual tumors (mm²).

Application of TLR ligands

The fully PTO-modified CpG oligonucleotide 1826 (5'-TCCATGACGTTCTGACGTT-3') (Coley Pharmaceutical Group) was injected subcutaneously either peritumorally or in the contralateral flank (100 µg CpG in PBS). EG-7 tumor-bearing mice were injected with 10 µg CpG complexed with 50 µg of liposomal transfection reagent DOTAP (Roche). Poly (I:C) (Amersham Bioscience) was applied intraperitoneally (250 µg). LPS (Sigma-Aldrich) and resiquimod (R848) (Alexis Biochemicals) were injected subcutaneously into the flank contralaterally to the tumor (20 µg).

Immunohistology and cell counting

To permit the evaluation of highly standardized tissue areas, tumor samples were bisected perpendicular to the skin at their widest diameter in order to obtain cryosections (5 µm) derived from the tumor center. The following primary antibodies were used: rat anti-mFoxP3 (Ebioscience), rat anti-mCD4 (Biolegend), Syrian hamster anti-mCD3, rat anti-mCD8. Cy3 goat anti-Syrian hamster IgG, biotinylated donkey anti-rat IgG, rhodamin red X streptavidin, alkaline phosphatase streptavidin (all Jackson ImmunoResearch) and Alexa-488 or -633 streptavidin (Invitrogen) were used as detection reagents. Infiltrating cells were assessed by systematically counting non-overlapping visual fields (high power fields, hpf) of the entire tumor section using a fluorescence microscope equipped with an 40x oil immersion objective (Carl Zeiss). Necrotic areas and blood vessels were excluded from evaluation. Counting was performed blinded by two independent investigators. Immunohistology on human paraffin-embedded sections was performed on biopsy tissue of breast cancer tissue that was retrieved from the archives of the Institute of Pathology of the Ludwig Maximilian University, Munich, Germany. Age of patients ranged from 26 to 80 years. All patients had been treated surgically between 2004 and 2008 at the same institution (Department of Obstetrics and Gynecology Maistrasse, University of Munich). Tissue was stained with the following unconjugated primary antibodies and the respective isotype control antibodies: rabbit anti-hCCL22 (Peprotech), mouse anti-hCD14

(Novocastra), mouse anti-hCD68 (Dako), rat anti-hDC-LAMP (Dendritics), mouse anti-hDC-SIGN (Abcam) and purified rabbit IgG (Biozol). For detection, the following secondary antibodies were used: biotinylated goat anti-rabbit (Vector), Cy2 goat anti-rat, and Alexa Fluor 488 donkey anti-mouse (both Jackson ImmunoResearch). For imaging, a confocal laser scanning microscope (LSM 510, Carl Zeiss) was used and images were processed using Adobe Photoshop for adjustment of contrast and size.

Flow cytometry and adoptive T cell transfer

Tumors were mechanically disrupted, incubated with 1 mg/ml collagenase and 0.05 mg/ml DNase (both Sigma Aldrich) and passed through a cell strainer. Single cell suspensions were resuspended in 44 % Percoll (Biochrome) and layered over 67 % Percoll prior to centrifugation at 800 g for 30 minutes. Lymphocytes from the interphase were stained with anti-CD3-PerCP, anti-CD4-APC, anti-CD103-Fitc (all BD Biosciences) and anti-CCR4-Pe (Biolegend) followed by intracellular detection of FoxP3 using either anti-FoxP3-PE or anti-FoxP3-Pacific Blue antibody and premixed regulatory T-cell staining reagents (Ebioscience). Events were measured on a FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar). For adoptive transfer, single cell suspensions from the spleen and lymph nodes of healthy mice were labeled with CFSE (Invitrogen) according to the manufacturer's instructions and injected intravenously (3×10^7 cells per recipient mouse) into tumor-bearing recipients (mean tumor size 80 mm²).

Cytokine assays of tissue lysates

Tissue homogenates were resuspended in lysis buffer (BioRad Laboratories) and centrifuged. Total protein concentration was measured by Bradford assay (BioRad Laboratories). All samples were diluted to a protein concentration of 10 mg/ml and CXCL12, CCL21, CCL17 and CCL22

concentrations were measured by ELISA (R&D Systems). The final cytokine concentration was calculated as ng cytokine per g protein in the respective lysate.

Isolation of tumor-infiltrating leukocytes from human ovarian cancer

Pieces of freshly isolated ovarian cancer specimens resected by open surgery were mechanically disrupted, incubated with 1 mg/ml collagenase and 0.05 mg/ml DNase (both Sigma Aldrich), washed and incubated in PBS containing 2 mM EDTA for 20 minutes. After washing, a second incubation with collagenase and DNase was performed before passing the material through a cell strainer. The obtained single cell suspension was washed and used for cell culture or magnetic cell separation. The study was approved by the local ethics committee.

In vitro TLR stimulation of tumor-infiltrating cells and in vitro AH1-antigen stimulation

Tumor-infiltrating cells were isolated as described above and cells were treated with 5 µg/ml CpG 1826 (Coley Pharmaceutical Group), 1000 U/ml murine IFN- α (R&D Systems), 50 ng/ml murine IFN- γ (Peprotech) or 10 ng/ml murine IL-1 β , IL-2, IL-6, IL-10 or IL-12 (all Peprotech). Human tumor cell lines and single cell suspensions derived from ovarian cancer specimens were treated with 100 ng/ml human IFN- γ (Peprotech), 5 µg/ml CpG 2006 (Invivogen), 0.5 µg/ml R848 (Alexis Biochemicals) or transfected with 1000 ng/ml poly (I:C) (Invitrogen, Carlsbad, CA), 1000 ng/ml 5'-triphosphate RNA (5'-GCAUGCGACCUCUGUUUGA-3') (synthesis with the Megashortscript kit, Ambion Life Technologies) or with 1000 ng/ml of a non-stimulating control RNA (5'-AAAAAAAAAAAAAAAAAAAA-3') (CureVac). Prior to cell transfection poly (I:C) and the RNA was complexed in 50 ml Opti-MEM medium (Invitrogen) containing 1% Lipofectamine 2000 (Invitrogen). To quantify antigen specificity tumor-infiltrating T cells were stimulated with AH1 peptide (2 µM, SPSYVYHQF, Anaspec) for 4 hours in presence of Brefeldin A (5 µg/ml), fixed and permeabilized (eBioscience Staining Buffer Set) and incubated with anti-IFN- γ -PE-Cy7 antibody (Biolegend).

Cell sorting

Microbead-based CD11c and CD8 T-cell isolation kits (Miltenyi Biotec) were used to isolate highly pure DCs (>95%) and CD8 T cells (>90%) from single cell suspensions of subcutaneous tumors. APC-labeled anti-CD11c antibody (Biolegend) was used to confirm DC purity via FACS analysis. To isolate human CD14⁺ cells CD14 microbeads were used (Miltenyi Biotec).

RNA isolation and quantitative PCR analysis of tumor-infiltrating cells

Total RNA was extracted from tumor-infiltrating cells using High Pure RNA Isolation Kit (Qiagen). 1 µg of RNA was converted to cDNA using the Revert Aid First strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR amplification was performed with the Light Cyclers TaqMan Master (Roche Diagnostics) on a LightCycler 2.0 instrument (Roche Diagnostics) together with the Universal Probe Library System (Roche Diagnostics). Relative gene expression is shown as a ratio of the expression level of the gene of interest to that of hypoxanthine phosphoribosyltransferase (HPRT) RNA. Primers were obtained from Metabion.

Statistical analysis

All data are presented as mean \pm SEM and were analyzed as appropriate by unpaired Student's t-test or by ANOVA test using the SNK correction. Correlations were analyzed two-tailed by Pearsons correlation. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software).

Results

Innate immune stimulation reduces tumor infiltration by Treg

To investigate the effect of PRR stimulation on tumor infiltration by Treg, we quantified these cells in CT26 tumors from mice treated with the TLR9 ligand CpG. Intratumoral Treg and total CD3⁺ T cells were visualized by immunofluorescence and counted according to a highly standardized protocol (see material and methods section). A majority of tumors from untreated mice showed prominent Treg cell infiltrations with formation of focal clusters (analysis of 384 visual fields from tissue sections of eleven individual tumors) (**Fig. 1 a, b**). In mice treated with CpG, Treg cells were almost completely absent from the tumors (analysis of 160 visual fields from twelve tumors). This effect was observed both in tumors treated with CpG peritumorally and upon CpG injection distant from the tumor. The decrease was selective for Treg, as total infiltrating CD3⁺ T cells were in contrast increased after CpG treatment (**Fig. 1 a, b**). Selectivity for Treg was also shown within the CD4⁺ T cell population as indicated by a decreased FoxP3 to CD4 ratio (**Supplementary Fig. 1**). Using flow cytometry we confirmed the decrease of intratumoral Treg, demonstrated by a reduction in FoxP3⁺ cells per gram tumor tissue and a proportional decrease within the CD3 and CD4 T cells (**Fig. 1c and Supplementary Fig. 1**). Four days after a single CpG injection the absolute number of intratumoral Treg was still strongly decreased. Treg numbers returned to the level of untreated mice eight days after one CpG injection, although the Treg to CD8 ratio remained strongly decreased (**Supplementary Fig. 1c**). Decreased Treg cell infiltration was not limited to the CT26 model, as a reduction in tumor-infiltrating Treg cells was observed upon CpG treatment in two additional tumor models in mice of different genetic background (**Supplementary Fig. 2**). To test whether suppression of intratumoral Treg infiltration can be mediated by activation of other PRR, we injected mice with the MDA-5 and TLR3 ligand poly(I:C) (17), the TLR7 ligand R848 and the TLR4 ligand LPS. Infiltrating FoxP3⁺ and CD3⁺ cells were quantified in 750 visual fields of 23 tumors. As seen

after CpG application, treatment with poly(I:C) and R848 led to a striking reduction in tumor-infiltrating FoxP3⁺ cells with a strong decrease of the FoxP3 to CD3 ratio (**Fig. 1d**). No significant reduction was seen upon treatment with LPS, which may be due to the fact that LPS also induces Treg proliferation (18). In conclusion, PRR activation with CpG, poly(I:C) or R848 clearly and selectively decreases the number of Treg within tumor tissues.

Innate immune activation inhibits the migration of regulatory T cells into the tumor

To examine whether the decreased numbers of intratumoral Treg result from a reduced migration of FoxP3⁺ cells into the tumor, we analyzed the trafficking of adoptively transferred Treg in tumor-bearing mice that had been treated with CpG. Mice bearing established CT26 tumors received one injection of CpG. 24 hours later CFSE-labeled cells from the spleen and lymph nodes of healthy donor mice were transferred intravenously. Tumors were resected 18 hours after transfer and CFSE⁺ T cells were analyzed by flow cytometry (**Fig. 2a**). Strikingly, in the tumors of CpG-treated animals the fraction of transferred Treg within intratumoral CFSE⁺ CD3⁺ T cells was reduced on average almost 4-fold compared to untreated mice (**Fig. 2a,b**). In contrast, no difference in the proportion of transferred Treg was observed in the spleen. Within the intratumoral CD4⁺ T cells, the proportion of transferred FoxP3⁺ Treg was also reduced whereas no decrease was observed for FoxP3^{neg} T effector cells (**Fig. 2c**), indicating a specific inhibition of Treg migration. In conclusion, these findings demonstrate that CpG suppresses the migration of FoxP3⁺ Treg into the tumor.

Innate immune activation suppresses tumor-associated CCL22 in mice and humans

The migration and retention of Treg in peripheral tissues is mediated by the interaction of specific integrins and chemokine receptors with their respective ligands (19). To characterize the molecular mechanisms responsible for the decrease in Treg migration into the tumor upon treatment with TLR ligands, we analyzed the expression of several Treg-related chemokines and

chemokine receptors after TLR stimulation of tumor-bearing mice. Two receptors expressed on Treg are critically involved in the recruitment of these cells: the chemokine receptor CCR4 (3) and the integrin CD103 that mediates retention of Treg within peripheral tissues (20). Treatment of tumor-bearing mice with CpG did not modify the expression of CCR4 or CD103 on FoxP3+ cells (**Fig. 3a**). Several chemokines such as CCL5, CCL17, CCL21, CCL22 and CXCL12 have been described to mediate homing of Treg in different circumstances (19,21-23). In addition, the chemokine CCL22 has been associated with homing of Treg to malignant tumors (1,19). We analyzed intratumoral levels of all these chemokines in CT26 tumors of CpG-treated mice by ELISA. Strikingly, we found a strong suppression of CCL22 levels in the tumor tissue upon treatment with the TLR ligand (**Fig. 3b**). A decrease was also observed for CCL17, although this chemokine was expressed only at very low levels. No suppression was seen for CCL5, CCL21 or CXCL12 (**Fig. 3b**). Substantial intratumoral levels of CCL22 were observed in three other subcutaneous murine tumor models and treatment with CpG decreased intratumoral CCL22 in all three models (**Fig. 3c**). Further, suppression of intratumoral CCL22 was also seen upon injection of poly(I:C), R848 or LPS, showing that this also results from activation of other PRR (**Fig. 3d**).

Interestingly, we observed a linear correlation between intratumoral CCL22 and the number of infiltrating FoxP3+ cells (**Supplementary Fig. 3**). To examine whether the reduction of intratumoral Treg by TLR ligands is due to suppression of CCL22, we established a CT26 tumor cell line with inducible expression of CCL22 (CT26-CCL22^{dox}). In this model, exposure of the tumor cells to doxycycline induces secretion of CCL22 (**Supplementary Fig. 4a**). When CT26-CCL22^{dox} cells were implanted subcutaneously in mice, addition of doxycycline to the diet of the animals led to increased intratumoral CCL22 production and enhanced infiltration of tumors with Treg (**Supplementary Fig. 4b**). Doxycycline-dependent induction of CCL22 expression in these tumors was used to prevent the suppression of intratumoral CCL22 by TLR ligands. Indeed, no

suppression of CCL22 was observed upon treatment with CpG in mice bearing subcutaneous CT26-CCL22^{dox} tumors that received doxycycline (**Fig. 3e, left panel**). Strikingly, in this group of mice no reduction of intratumoral Treg was observed, demonstrating that suppression of CCL22 is indeed responsible for the inhibition of Treg migration upon TLR stimulation (**Fig. 3e, right panel**).

We next examined whether suppression of tumor-associated CCL22 is also a phenomenon that can be observed in humans. We prepared single cell suspensions from fresh human ovarian cancer samples and incubated these with ligands for different PRR. High levels of CCL22 were detected in the untreated culture supernatants, whereas application of R848, poly(I:C) and triphosphate RNA as ligand for RIG-I suppressed CCL22 (**Fig. 3f**). Interestingly, CCL22 suppression in the human tumor supernatants was absent upon stimulation with CpG (**Fig. 3f**). This is probably due to the more restricted expression pattern of the receptor for CpG, TLR9, in humans. Thus, PRR activation suppresses cancer-associated CCL22 in both mice and humans.

Dendritic cells and macrophages produce CCL22 in murine and human tumors

To elucidate the mechanisms of CCL22 suppression we next aimed to identify the source of intratumoral CCL22. Culture supernatants of seven different murine tumor cell lines including the CT26, B16, EG-7 and panc02 cells were uniformly negative for CCL22, indicating that the tumor cells themselves do not produce the chemokine (**Fig. 4a**). As in secondary lymphatic organs of mice CD11c⁺ dendritic cells (DC) are the producers of CCL22 (24), we hypothesized that these cells could also be responsible for the production of CCL22 in tumors. To test this hypothesis we isolated CD11c⁺ cells from subcutaneous tumors. Expression of CCL22 was observed almost exclusively in the CD11c-positive fraction, indicating that tumor-infiltrating DC are the source of intratumoral CCL22 in mice (**Fig. 4b**). As a previous study (25) suggested a potential role for CD8⁺ intratumoral T cells as source of CCL22, we also isolated CD8 T cells from the tumors but

could not detect CCL22 expression in this fraction (**Fig. 4b**). We further measured CCL22 levels in the supernatant of several human cancer cell lines. No detectable CCL22 levels were seen in the majority of samples, except for two breast cancer and one melanoma cell line that secreted CCL22 (**Fig. 4c**). Since CCL22 secretion has been reported upon stimulation with IFN- γ in several breast cancer cell lines (26), we stimulated all human cell lines with IFN- γ . This indeed enhanced the chemokine production of the CCL22-secreting breast cancer and melanoma cells (CAMA-1, MCF-7 and melanoma A431), but CCL22 secretion remained undetectable in all other cell lines tested (**Supplementary Fig. 5**). As CCL22 was not secreted by the majority of human cancer cells tested, it appeared likely that, as in mice, tumor-infiltrating immune cells were responsible for the production of intratumoral CCL22. We performed immunohistology of ovarian and breast cancer tissues and found CCL22 to be expressed by cells with macrophage-like morphology (**Fig. 4d**). We therefore isolated CD14-positive cells from single cell suspensions of fresh ovarian cancer specimens, cultured the cells for 3 days and determined CCL22 levels in the supernatants. CCL22 production was restricted to the CD14-positive fraction, demonstrating that these cells represent the main source of CCL22 in these human tumors (**Fig. 4e**). Double immunofluorescence imaging revealed co-localisation of CCL22 with CD14 and CD68, confirming the expression by macrophages (**Fig. 4f**). Interestingly, some mature DC, characterized by staining of DC-LAMP, were also positive for CCL22, whereas no co-localisation was shown for DC-SIGN, a marker for more immature DC (**Fig. 4f**). In conclusion, intratumoral CCL22 is secreted by infiltrating dendritic cells and macrophages rather than by the tumor cells themselves.

Suppression of intratumoral CCL22 is mediated by type I interferon

In order to investigate the mechanism of CCL22 suppression we measured the secretion of this chemokine by tumor-infiltrating immune cells under different culture conditions. First we found that culture supernatants from TLR-stimulated splenocytes (the TLR ligand itself was removed

by washing 2 hours after stimulation) suppressed CCL22 secretion by freshly isolated tumor-infiltrating immune cells, suggesting that a secreted factor was responsible for CCL22 suppression (data not shown). To identify this factor, we incubated the tumor-infiltrating immune cells with a set of recombinant cytokines that are typically released upon innate immune activation (27). A minor decrease of CCL22 was seen upon addition of IL-10 and IFN- γ and no change occurred with IL-1, IL-2, IL-6 and IL-12 (data not shown). In contrast, potent suppression of CCL22 was seen upon treatment of the tumor-infiltrating cells from CT26, B16 and Panc02 tumors with IFN- α (**Fig. 5a**). CCL22 secretion was also suppressed by IFN- α in isolated tumor-derived CD11c⁺ cells, indicating that DC are directly targeted by IFN- α (**Fig. 5b**). To evaluate the contribution of IFN- α to the suppression of CCL22 by innate immune activation, we induced tumors in interferon receptor-deficient mice (IFNAR) and treated these animals by PRR stimulation. Strikingly, no suppression of intratumoral CCL22 and tumor growth was seen in IFNAR mice (**Fig. 5c and Supplementary Fig. 6a**), demonstrating that type I interferon is a key mediator in the process of CCL22 suppression. Treatment of tumors with recombinant IFN- α tended to reduce intratumoral CCL22 and Treg numbers, but did not significantly suppress them, indicating a higher efficacy of PRR-induced IFN- α (**Supplementary Fig. 6b**).

CCL22 suppression is required for effective anti-tumor immunotherapy

The outcome of anti-tumor treatment with CpG is dependent on an effective CD8⁺ T cell response (9) and (**Supplementary Fig. 7**). We examined the contribution of the suppression of intratumoral CCL22 to this therapeutic effect of CpG. We treated mice bearing CT26-CCL22^{dox} tumors with CpG and simultaneously prevented suppression of CCL22 by feeding with doxycycline. As expected, in the absence of doxycycline a clear suppression of tumor growth was observed in the animals receiving CpG treatment (**Fig. 6**). In contrast, CpG treatment did not significantly block tumor growth when intratumoral CCL22 secretion was maintained by

doxycycline administration. Thus, we demonstrate that suppression of CCL22 is necessary for the therapeutic efficacy of TLR ligands.

Discussion

Tumor-infiltrating Treg inhibit anti-tumor immunity (6) and are associated with poor prognosis in several types of human cancer (1,2). In the present study we show that stimulation of innate immunity through TLRs and RIG-I-like helicases strongly reduces the absolute number of intratumoral FoxP3⁺ Treg in several mouse tumor models in a highly selective manner. Indeed, the number of FoxP3-negative effector CD8⁺ T cells within the tumor was even increased. This is in accordance with previous reports showing higher proportions of T effector cells upon treatment with CpG (28). The TLR7 agonist imiquimod increased the T effector cell to Treg ratio in human squamous cell cancer, but did not alter the proportion of antigen-specific T effector and Treg cells in the lymph node in a murine anti-tumor vaccine model (29,30). Interestingly, a suppression of Treg infiltration upon treatment with CpG has been reported in a model of leishmania infection of the skin: TLR stimulation of the animals reduced the number of FoxP3⁺ cells in the infected skin and thus led to clearance of the pathogen (31). We show here that suppression of intratumoral Treg by TLR agonists results from a blockade of Treg immigration into the tumor. This is to our knowledge the first report demonstrating a direct inhibition of Treg migration upon innate immune activation. Taken together with the observation that TLR stimulation suppresses Treg infiltration in leishmania infection, these findings suggest that activation of the innate immune system through TLRs may generally prevent Treg migration to sites of inflammation. The local reduction of Treg in response to microbial patterns could thus permit an efficient response against pathogens within the affected tissues.

It was previously demonstrated that ligands for PRR and cytokines such as IFN- α affect immunosuppression exerted by Treg. For instance, activation of TLR7 and TLR9 on dendritic cells renders effector T cells refractory to Treg suppression, whereas stimulation with IFN- α or ligands for TLR2 and MDA-5 expressed by Treg can directly inhibit their suppressive function or

suppress their proliferation (15,32-34). We describe here the blockade of Treg migration as a novel mechanism of action in the process of innate immune activation by ligands for PRR. We further show that inhibition of Treg migration indeed contributes to tumor regression. The efficacy of modulating Treg and T effector cell activity for immune activation against cancer has recently been highlighted by the success of the CTLA-4 blocking antibody ipilimumab (35). Further, blockade of the Treg-associated receptor CCR4 by a small molecule enhanced tumor immunity (36). Thus, inhibition of Treg migration represents an interesting future concept of anti-cancer immunotherapy.

An analysis of several Treg-associated chemokines and homing receptors during immune activation revealed a potent suppression of intratumoral levels of CCL22, a chemokine that is crucial for the recruitment of Treg to tumors: In mice, CCL22-mediated Treg migration has been shown in a model of lung cancer (37) and in humans, high levels of intratumoral CCL22 have been observed in many types of cancer (1,2,26,38). The cellular source of CCL22 in the tumor tissue is however still unclear. We show here that, with the exception of some types of breast cancer, CCL22 is secreted not by the tumor cells or by tumor-infiltrating T cells as previously reported (25), but by tumor-infiltrating DC and macrophages. Interestingly, it has been generally assumed that CCL22 secretion is enhanced rather than suppressed upon TLR stimulation (39,40). This assumption is however based on observations made using DC differentiated in vitro (39,40), in which we also observed enhanced CCL22 secretion upon TLR stimulation (data not shown). In primary cells isolated from murine or human tumors, however, we clearly show that CCL22 secretion by DC and macrophages is suppressed upon stimulation with CpG and other activators of innate immunity.

Suppression of CCL22 upon PRR stimulation was dependent on type I interferon. The concept of CCL22 suppression by type I interferon is supported by previous reports where IFN- α -

containing combinations were used: CCL22 was reduced in CD3-stimulated T cells upon treatment with IL-12 and IFN- α (41), in peripheral human blood-derived DC upon incubation with an IFN- α -containing maturation cocktail (42) and in tumor cell cultures through a COX-1 inhibitor and IFN- α (43). We clearly demonstrate here that IFN- α as a single agent directly suppresses CCL22 secretion of tumor-infiltrating immune cells. Although TLR-induced CCL22 suppression critically depends on IFN- α , treatment with recombinant IFN- α alone was not as efficient as TLR ligands for CCL22 suppression, a phenomenon that could be explained by the different pharmacokinetics of these agents. A major contribution of IFN- α to the therapeutic efficacy of TLR ligands such as CpG or polyI:C was shown previously and could be confirmed here (44,45) (**Supplementary Fig. 6**). We now demonstrate that the type I interferon-dependent CCL22 suppression contributes to the anti-cancer effect of innate immune activation. Indeed, cancer therapy with IFN- α is established for melanoma and can also be used for other types of human cancer (8). Our findings uncover CCL22 suppression as a so far unknown mechanism of action of anti-cancer therapy with IFN- α . This has important therapeutic implications as patients with high intratumoral CCL22 may derive a higher benefit from IFN- α treatment. It will be interesting to investigate intratumoral CCL22 as a predictive biomarker for interferon treatment. In summary, our findings identify inhibition of Treg migration by CCL22 suppression as a so far unknown mechanism of innate immune activation with important implications for cancer therapy.

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Figure legends

Figure 1. Ligands for TLR and RLR prevent tumor infiltration by Treg.

Mice bearing CT26 tumors with an average size of 60 mm² were treated three times at two-day intervals with CpG, either peritumorally (p.t., n = 8) or contralaterally (c.l., n = 4), poly(I:C) (n = 6), R848 (n = 6) or LPS (n = 5) or remained untreated (n = 18). Two days after the last injection, tumors were prepared for histology and stained for FoxP3 and CD3 or analyzed by flow cytometry. **(a)** Representative tissue areas of untreated and CpG-treated CT26 tumors, FoxP3+ (green) and CD3+ (red). **(b)** Infiltrating FoxP3+ and CD3+ cells were counted in each tumor sample as described in the Methods section. Each data point represents the number of positive cells in one visual field (0.25 mm²). Each data set (vertically aligned dots) represents a tumor from an individual mouse. Numbers at the bottom of each data set indicate the number of visual fields counted per tumor. Bars (—) indicate the mean cell count per visual field in one tumor. The ratio of FoxP3+ to CD3+ cells is indicated (histogram). **(c)** The absolute number of tumor-infiltrating CD4+ FoxP3+ Treg per gram tumor and the proportion of FoxP3+ cells within tumor-infiltrating CD3+ T cells were determined by flow cytometry. Data for one representative mouse per group and average of all mice per group (n = 7) are shown. **(d)** CT26 tumors from poly(I:C)-, R848- and LPS-treated mice were analyzed for FoxP3+ and CD3+ cell infiltration by immunohistology. Data are represented as in (b). Error bars indicate SEM. *P* values were calculated relative to untreated mice (**p* < 0.05; ***p* < 0.01).

Figure 2. Treatment with CpG inhibits migration of FoxP3+ cells into the tumor.

Mice bearing CT26 tumors (average size 80 mm²) were treated at day 21 after tumor induction by one peritumoral injection of CpG (n = 7) or remained untreated (n = 7). 24 hours later, the animals received an i.v. transfer of CFSE-labeled cells from the spleen and lymph nodes of healthy donor mice. Tumors were resected 18 hours after transfer, followed by flow cytometry

analysis of intratumoral and splenic CFSE⁺ T cells. **(a-b)** The proportion of CFSE⁺CD4⁺FoxP3⁺ Treg within the CFSE⁺CD3⁺ T cells was analyzed in the tumor and spleen of untreated and CpG-stimulated mice. Results are shown for **(a)** one representative mouse per group (in tumor) and **(b)** average of all mice per group (n = 7). **(c)** Analysis of the proportion of transferred FoxP3⁺ Treg cells within tumor-infiltrating CFSE⁺CD3⁺CD4⁺ T cells. Error bars indicate SEM. *P* values were calculated relative to untreated mice (***p* < 0.01).

Figure 3. TLR stimulation suppresses intratumoral CCL22 in mice and humans.

(a-b) CT26 tumor-bearing mice were treated with CpG as described in Figure 1 (n = 6 mice per group). **(a)** The expression of CD103 and CCR4 on CD4⁺FoxP3⁺ splenic Treg was assessed by flow cytometry 2 days after the last injection and **(b)** homogenates of the tumor tissue were analyzed for the indicated chemokines by ELISA. **(c)** Intratumoral CCL22 levels of CpG-treated or untreated subcutaneous B16 (n=7), EG-7 (n=5) and Panc02 (n=7) tumors were measured by ELISA in tissue homogenates. **(d)** CT26 tumor-bearing mice were treated with poly(I:C), R848 or LPS as in Figure 1 and tumors were analyzed for CCL22 levels by ELISA (n = 7 mice per group). **(e)** Mice (n = 7 per group) bearing subcutaneous CT26-CCL22^{dox} tumors were fed with a normal or doxycycline-containing diet. At day 21 after tumor induction both groups were either treated with CpG (three times at a three-day interval) or remained untreated. One day after the last injection, tumors were analyzed for intratumoral CCL22 and FoxP3⁺ T cells by ELISA and flow cytometry, respectively. The levels of CCL22 and Treg in mice with untreated CT26-CCL22^{dox} tumors was set to 100%. **(f)** Single cell suspensions from freshly isolated human ovarian cancer specimens were incubated with CpG, R848, poly(I:C), 5'-triphosphate RNA (3p-RNA), a non-activating control RNA or remained untreated. Error bars indicate SEM. *P* values were calculated relative to untreated samples (**p* < 0.05; ***p* < 0.01; ns, not significant).

Figure 4. Dendritic cells and macrophages produce CCL22 in murine and human tumors.

(a) Culture supernatants of murine tumor cell lines and bone marrow-derived dendritic cells (BM-DC, used as positive control) were analyzed for secretion of CCL22 by ELISA. **(b)** CD11c⁺ cells and CD8⁺ T cells were isolated from subcutaneous CT26 and B16 tumors and CCL22 mRNA levels were quantified by qRT-PCR. **(c)** CCL22 levels in the culture supernatants of different human tumor cell lines were measured by ELISA. **(d)** Representative examples of human breast cancer tissue sections were stained for CCL22 (brown cells). **(e)** CD14⁺ cells isolated from single cell suspensions of freshly dissected ovarian cancer specimens were cultured for 3 days (n = 4 patients). CCL22 was then measured in the culture supernatants. **(f)** Ovarian and breast cancer tissues were prepared for histology and stained for CCL22 (red) and CD14, CD68, DC-LAMP, DC-SIGN (green) or with respective isotype controls. Error bars indicate SEM. *P* values of isolated CD11c⁺ and CD14⁺ cells were calculated relative to the CD11c⁻ and CD14-depleted fraction, respectively (**p* < 0.05; ***p* < 0.01).

Figure 5. Suppression of intratumoral CCL22 is mediated by type I interferon.

(a) Tumor-infiltrating cells from subcutaneous CT26, B16 and Panc02 tumors and **(b)** CD11c⁻ positively sorted single cell suspensions from Panc02 tumors were treated with IFN- α or remained untreated. After incubation for three days, CCL22 levels in the supernatants were determined by ELISA. **(c)** Subcutaneous B16 tumors were induced in C57BL/6 (n = 10) or type I interferon receptor-deficient (IFNAR) mice (n = 10). At day 21 after tumor induction both groups were either treated with poly(I:C) (twice at a three-day interval) or remained untreated. Two days after the last injection, tumors were prepared and CCL22 levels in the tissue homogenates were measured by ELISA. Error bars indicate SEM (**p* < 0.05; ***p* < 0.01).

Figure 6. CCL22 suppression is required for the therapeutic efficacy of CpG.

BALB/c mice were inoculated with subcutaneous CT26-CCL22^{dox} tumors and fed with a normal (n = 28) or doxycycline-containing (n = 28) diet. At day 21 after tumor induction both groups

were either treated with CpG (three times at a three-day interval) or remained untreated. Tumor size was measured every second day and expressed as mm². Error bars indicate SEM. *P* values were calculated for day 30 (**p* < 0.05; ns, not significant).

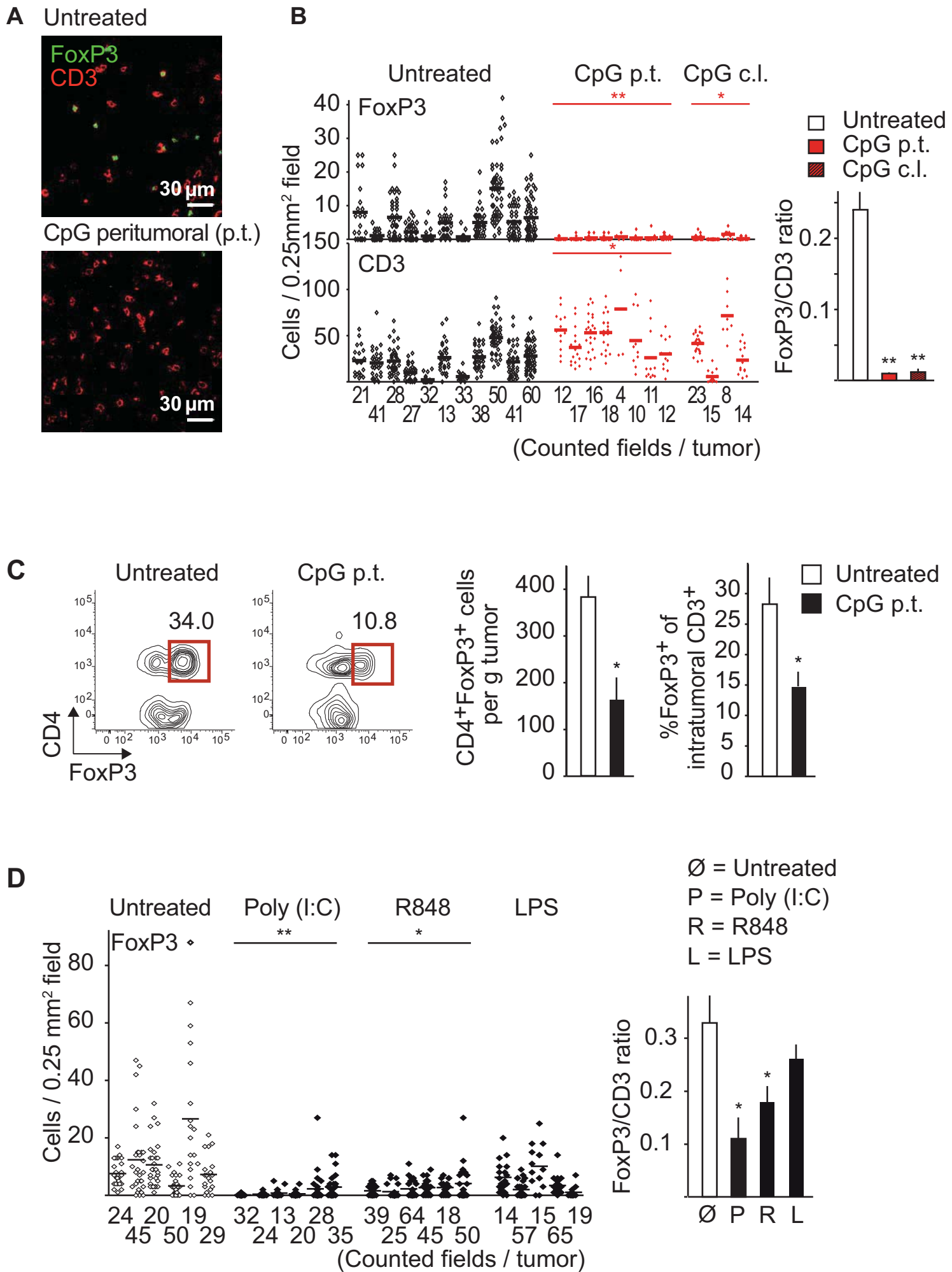


Figure 1

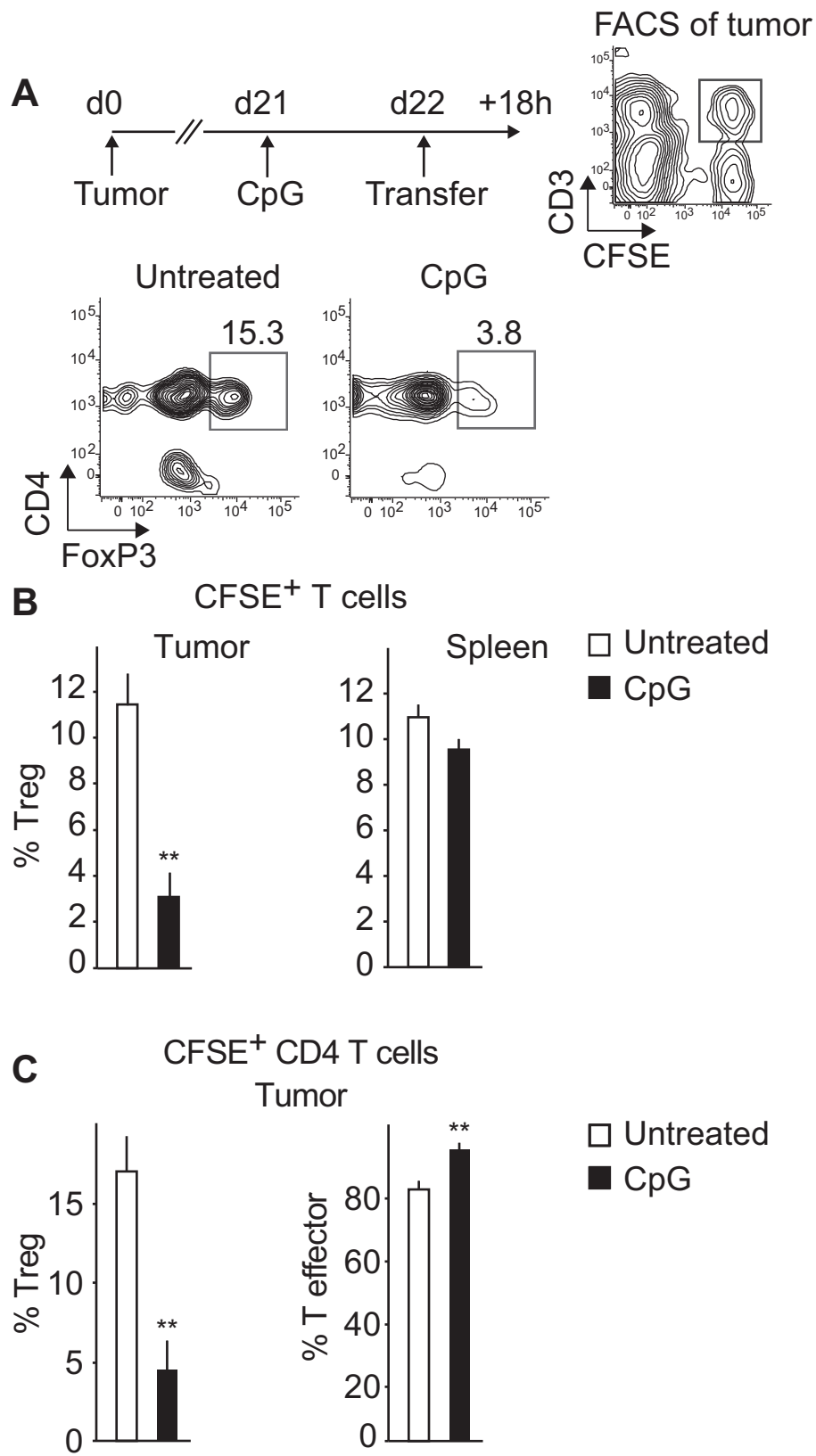


Figure 2

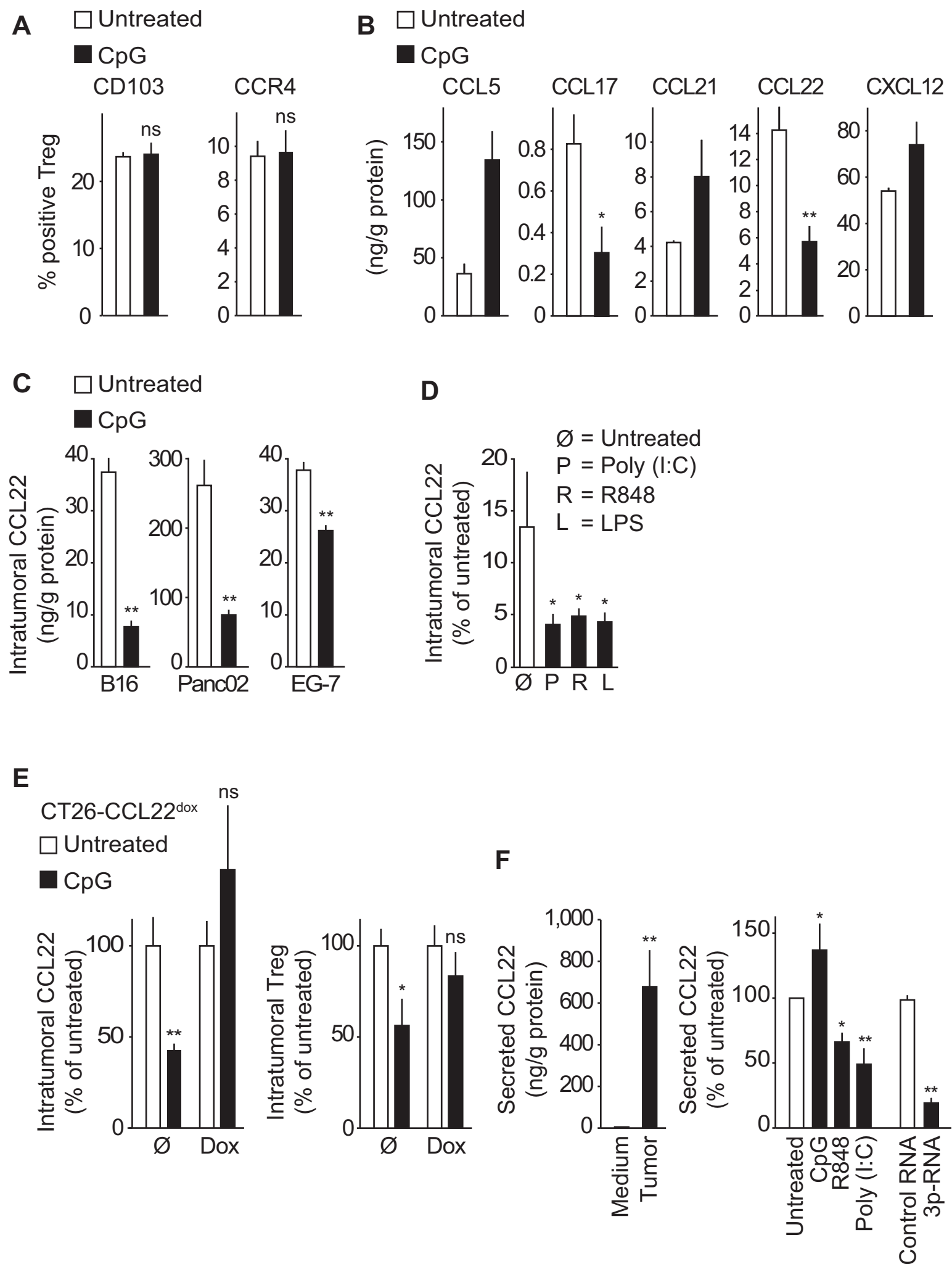


Figure 3

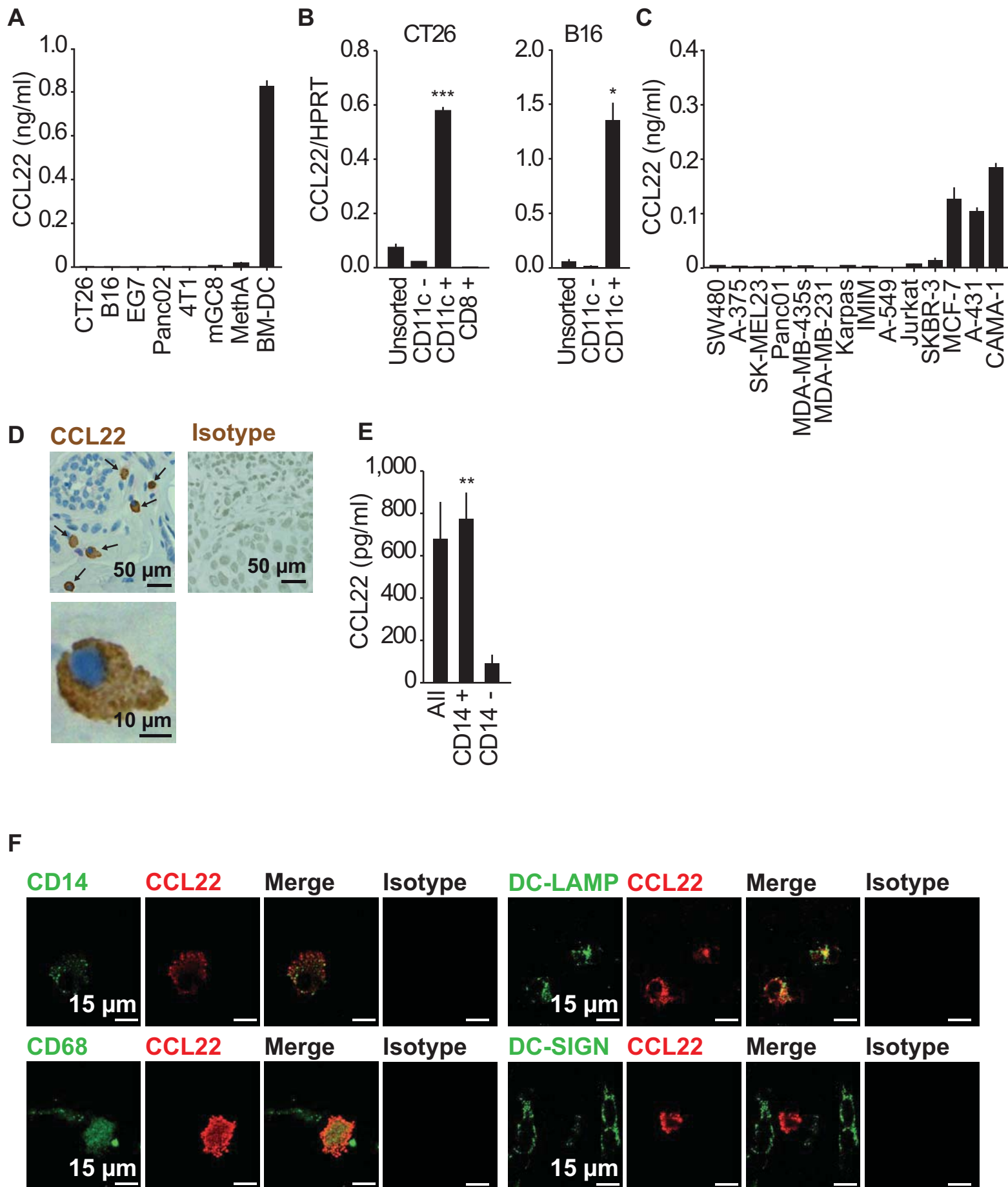


Figure 4

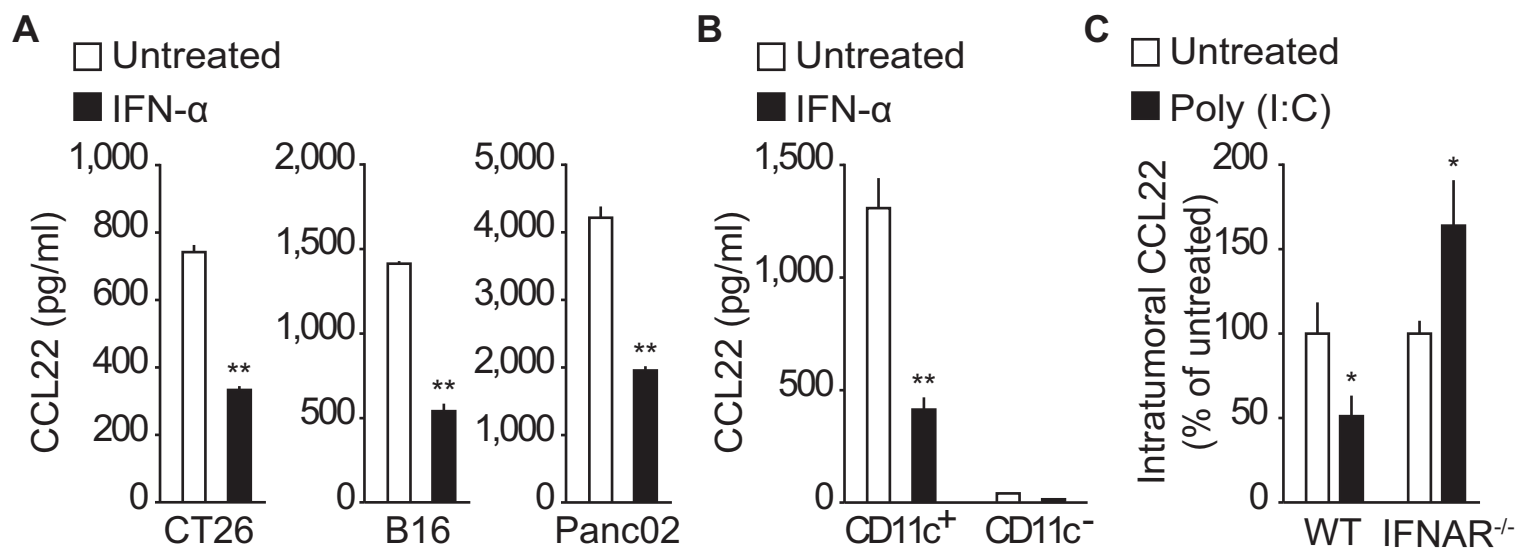


Figure 5

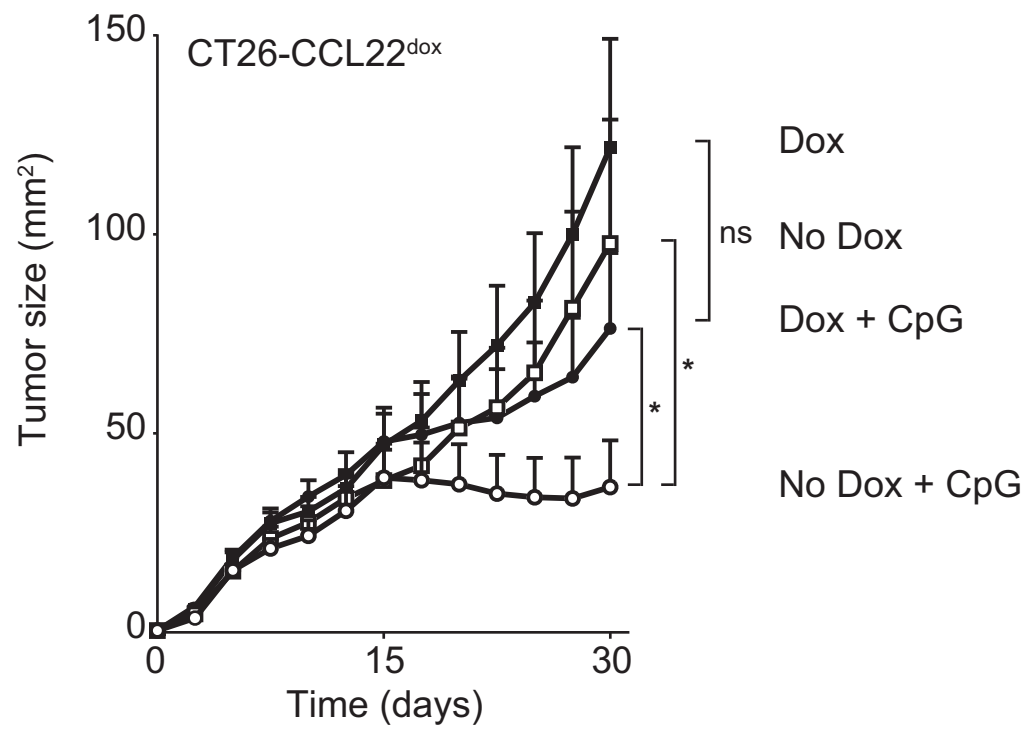


Figure 6